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RXLR effectors of plant pathogenic oomycetes

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Oomycetes are a phylogenetically distinct group of organisms that include some of the most devastating plant pathogens. Recent characterization of four oomycete *Avr* genes revealed that they encode effector proteins with a common modular structure, including a N-terminal conserved RXLR motif. Several lines of evidence initially indicated, with support from more recent works, that these Avr proteins are secreted by the pathogen and then translocated into the host cell during infection. In addition to elucidating the machinery required for host-cell transport, future works remain to determine the myriad virulence functions of oomycete RXLR effector proteins.

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Introduction

Prokaryotic and eukaryotic pathogens of plants secrete effector proteins to different cellular compartments of their hosts to modulate plant defense circuitry and enable parasitic colonization [1–4]. The current paradigm in the study of plant–microbe interactions is that unravelling the molecular function of effectors is central to a mechanistic understanding of pathogenicity. Indeed, significant progress has been made in elucidating the virulence functions of bacterial effectors [2], and research with eukaryotic plant pathogens is progressing rapidly as illustrated by the recent identification of effectors from the flax rust and barley powdery mildew fungi [5–7], the oomycetes *Phytophthora* and *Hyaloperonospora* [8,9,10••,11], as well as root-knot nematodes [12,13].

Oomycetes form a distinct group of eukaryotic microorganisms that includes some of the most notorious pathogens of plants [14]. Research on oomycete effectors has accelerated in recent years partly because of the blossoming of genomic resources. Oomycetes are now thought to secrete hundreds of effector proteins that target two distinct sites in the host plant [1,3,15••]. Apoplastic effectors are secreted into the plant extracellular space, whereas cytoplasmic effectors are translocated into the plant cell, where they target different subcellular compartments [1,3]. Several apoplastic effectors contribute to counter-defense by inhibiting host enzymes, such as proteases and glucanases, that accumulate in response to pathogen infection [16–18]. By contrast, the biochemical activities of cytoplasmic effectors remain poorly understood. Oomycete cytoplasmic effectors have been discovered through their avirulence (Avr) function, that is, their ability to trigger hypersensitive cell death on host genotypes with corresponding disease resistance (*R*) genes [8,9,10••,11], but their function in plants lacking cognate *R* genes remains largely unknown [3].

This review summarizes recent findings on the structure and function of the RXLR class of oomycete cytoplasmic effectors [1,3]. These effectors function inside host cells and are characterized by a highly conserved region defined by the invariant sequence RXLR. This review will cover two main topics of RXLR effector research: trafficking and virulence function.

The RXLR sequence defines a conserved domain of oomycete Avr proteins

Four oomycete *Avr* genes have been described in the past three years: $ATR1^{NdWsB}$ and *ATR13* from the downy mildew *Hyaloperonospora parasitica* [8,10••], *Avr1b-1* from the soybean pathogen *Phytophthora sojae* [11], and *Avr3a* from *Phytophthora infestans* [8,9,10••,11]. All four Avr proteins contain within the N-terminal 60 amino acids a secretory signal peptide and a conserved domain featuring the motif RXLR, flanked by a high frequency of acidic (D/E) residues (Figure 1).

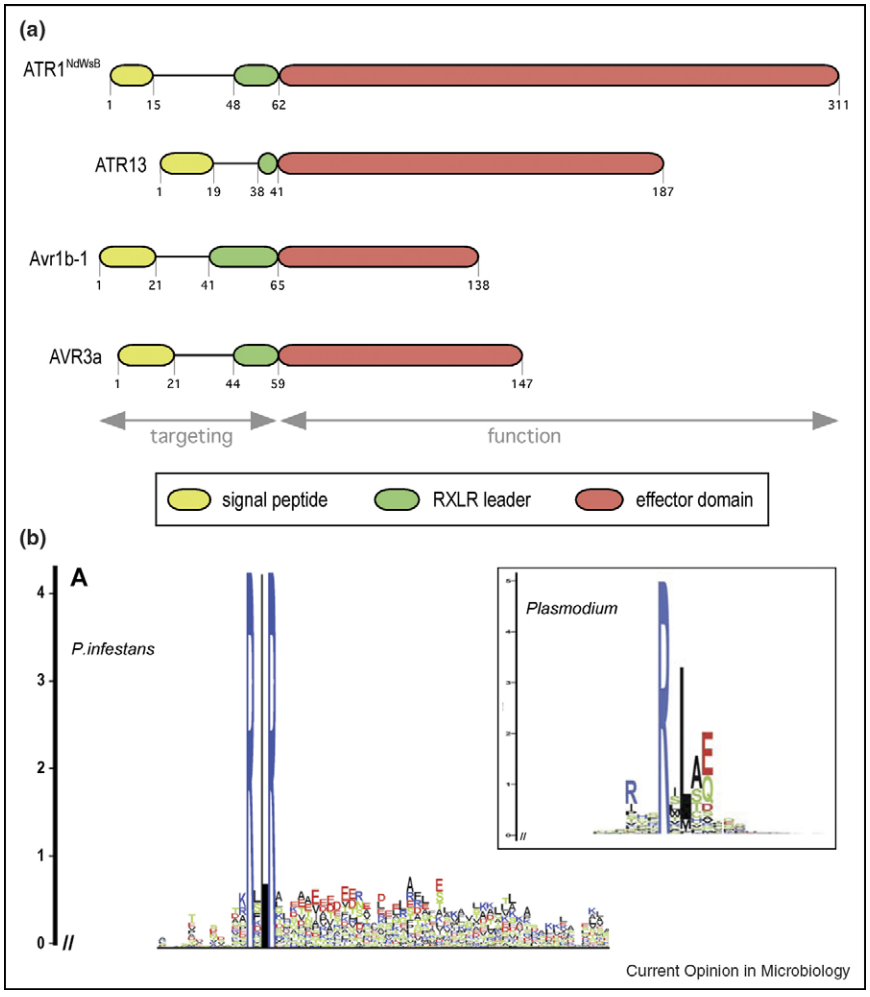
Oomycete RXLR proteins interact with intracellular host proteins

Interestingly, the R proteins that target each of these four Avr proteins belong to the intracellular class of NBS-LRR (nucleotide binding site and leucine-rich repeat domain) proteins [19–21]. The intracellular location of these R proteins suggests that these Avr proteins are detected inside the plant cytoplasm [8,9,10••]. Consistent with this idea, $AVR3a^{KI}$, $ATR1^{NdWsB}$, and ATR13 do not require a signal peptide sequence to trigger hypersensitivity when directly expressed *in planta* [8,9,10••].

The *Phytophthora* RXLR domain mediates host targeting in *Plasmodium*

The oomycete RXLR motif is similar in sequence and position to the host cell targeting signal (HT/Pexel motif)

Figure 1



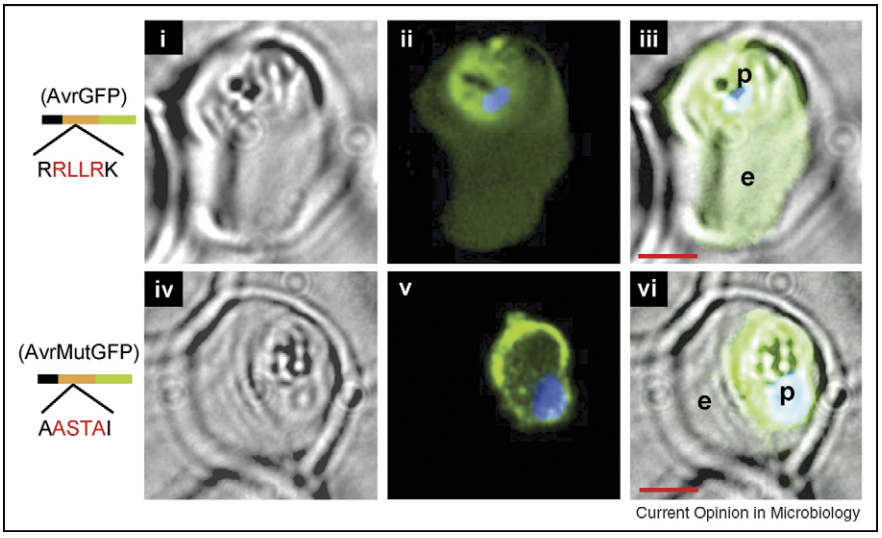
(a) Domain organization of cytoplasmic RXLR effectors. Schematic drawings of ATR1^{NdWsB} and ATR13 of *Hyaloperonsopora parasitica*, Avr1b-1 of *Phytophthora sojae*, and AVR3a of *Phytophthora infestans*. The numbers under the sequences indicate amino acid positions. The highlighted RXLR domain includes the RXLR sequence itself and the downstream dEER sequence. The gray arrows distinguish the regions of the effector proteins that are involved in secretion and targeting from those involved in effector activity. **(b)** Similarity between the RXLR motif of oomycetes and the HT/Pexel motif of *Plasmodium falciparum*. Sequence logos were derived from *P. infestans* and *P. falciparum* effector proteins. Adapted from Bhattacharjee *et al.* [24••].

required for translocation of proteins from malaria parasites (*Plasmodium* species) into host red blood cells [22,23]. The discovery that host-targeted proteins from *Phytophthora* and *Plasmodium* share a positionally conserved sequence begged the examination of functional conservation. Bhattacharjee *et al.* [24••] demonstrated that the RXLR leader sequences of *P. infestans* Avr3a and another candidate effector is sufficient to mediate the export of the green fluorescent protein (GFP) from the *Plasmodium falciparum* parasite to the host red blood cell (erythrocyte) (Figure 2). Mutations in the RXLR consensus abolished export. Consistent with the observed sequence biases flanking the RXLR motif (see above), regions upstream and downstream of the RXLR motif were required for host targeting, thereby defining a ca. 30 amino acid targeting domain. In summary, these findings suggest that plant and animal eukaryotic pathogens use similar signals for targeting effectors into host cells [24••]. However, it is presently unclear whether this functional similarity reflects conserved transport machinery between these divergent eukaryotes (see below for further discussion on this topic).

The RXLR domain is required for host cell targeting in *Phytophthora*

Recent evidence indicates that the RXLR motif is indeed required for targeting these oomycete effectors into host plant cells. It was recently found that the RXLR motifs of *P. infestans* Avr3a and *P. sojae* Avr1b-1 are needed to confer avirulence on resistant plants when expressed in

Figure 2



The *Phytophthora infestans* AVR3a RXLR leader region mediates the export of the green fluorescent protein (GFP) from the *P. falciparum* parasite to the host erythrocyte. Erythrocytes expressing wild-type or mutated RXLR region of AVR3a (residues 21–69) fused to a *P. falciparum* signal peptide and GFP. Panels (ii) and (v) represent fluorescence images, (i) and (iv) brightfield images, and (iii) and (vi) merged images. Parasite (p), erythrocyte (e), Hoechst stained nucleus (blue), scale bar represents 2 μm. Adapted from Bhattacharjee *et al.* [24••].

the pathogen, but not when transiently expressed *in planta* (Whisson *et al*. abstract 549; D Dou *et al*., abstract 552, XXIV Fungal Genetics Conference, Pacific Grove, CA, March 2007). Furthermore, an Avr3a fluorescent fusion protein was specifically secreted from haustorial projections, potentially accumulating inside host cells (S Whisson *et al*. abstract 549, XXIV Fungal Genetics Conference, Pacific Grove, CA, March 2007). By contrast, an RXLR mutant version of this fusion protein accumulated in the apoplastic space. These results suggest that the RXLR domain directs translocation of effector proteins into the plant host cell, following signal peptide-mediated secretion from the pathogen.

The RXLR domain is not required for effector activities

The emerging view then is that oomycete RXLR effectors, analogous to bacterial effectors of the type III secretion system, are modular proteins organized into two main functional domains [3] (Figure 1). The N-terminal domain, encompassing the signal peptide and RXLR region, functions in secretion and targeting, while the remaining C-terminal domain possesses the effector activity. This model predicts that the RXLR region should not be required for activity when the effector is expressed inside host cells. Indeed, Bos *et al.* [25••] recently showed that mutation of the *P. infestans* AVR3a^{KI} RXLR sequence did not interfere with induction of R3a hypersensitivity when the protein is directly expressed in *Nicotiana benthamiana* leaves. In fact, deletion analyses of AVR3a^{KI} showed that the C-terminal 75 amino acids, which excludes the RXLR region but includes the two polymorphic amino acids K^{80} and I^{103} that are mutated in the nonfunctional allele, was sufficient for avirulence function when expressed directly inside plant cells [25••]. These findings are consistent with the view that the N-terminal region of oomycete RXLR effectors is involved in secretion and targeting but is not required for effector activity.

The C-terminal domain of RXLR effectors is typically under positive selection

Direct interaction between an Avr protein and its cognate R protein can lead to a coevolutionary arms race [26]. As a result, the effector domains recognized by the R protein will be under selection to diversify. Consistent with a role in effector activity, the C-terminal regions of *H. parasitica* ATR1 and ATR13 have higher levels of polymorphisms, particularly non-synonymous substitutions, than the signal peptide and RXLR region [8,10••]. Furthermore, two out of the three polymorphic residues between the two *Avr3a* alleles of *P. infestans*, amino acids 80 and 103, are located in the C-terminal effector domain [9]. Recent genome-wide analyses of RXLR effectors showed that positive selection has for the most part targeted the C-terminal effector domain rather than the signal peptide and the RXLR regions (S Kamoun *et al*. abstract 188, XXIV Fungal Genetics Conference, Pacific Grove, CA, March 2007). In summary, the preponderance of non-synomonous polymorphisms in the C-terminal domain of the RXLR proteins is consistent with a role in effector activities, while the conservation of the N-terminal domains is consistent with a role in protein targeting.

Can RXLR effectors enter host plant cells in the absence of the pathogen?

Machinery for host cell transport of RXLR proteins could be encoded either by the pathogen, the host, or both. In our own work with AVR3a^{KI}, we noted that *in planta* expression of the full-length protein resulted in R3a-dependent hypersensitivity [25••]. Catanzariti *et al.* [5] reported similar results with the rust fungus effector AvrM. However, these experiments are not particularly informative since they could be equally explained by (1) secretion of the effector followed by its re-entry into the plant cell or (2) mis-targeting of the protein to the cytoplasm through the well-established retrograde transport pathway [27,28].

Shan *et al.* [11] reported that infiltration of *P. sojae* RXLR effector Avr1b-1, expressed in *Pichia pastoris*, into Rps1b soybean leaves resulted in cell death. The ability of the infiltrated protein to trigger cell death via its intracellular R protein, Rps1b, argues that pathogen-encoded machinery and structures (i.e. haustoria) are not required for host cell transport. Surprisingly, the ability of recombinant Avr1b-1 to trigger cell death presumably by entering plant cells has not been exploited to study the contribution of the RXLR domain for instance by infiltration of mutant forms of Avr1b-1. This is possibly explained by the poor reproducibility of these experiments. Indeed, recombinant Avr1b-1 produced in *Escherichia coli* or *Sf9* insect cells failed to exhibit any biological activity on Rps1b soybean plants (K Valer-Saldana, PhD thesis, Ludwig-Maximilians-University of Munich, 2006).

A model for RXLR effector delivery into the host

Many key questions remain about how the RXLR domain functions in host targeting of effectors. What is the transport machinery of RXLR effectors? Is it derived from the pathogen or are the effectors exploiting host transport systems? Is similar machinery used to deliver oomycete and *Plasmodium* effectors? Despite these persisting questions, some reasonable assumptions about the translocation process can be made. For instance, it seems sensible to break down the transport process into two steps [24••]. First, the effectors are secreted outside the pathogen cell through the general secretory pathway using endoplasmic reticulum (ER) type signal peptides. Then, the secreted effectors are transported across a host-derived membrane, most probably the haustorial membrane, via the RXLR leader. In the GFP export experiments of Bhattacharjee *et al.* [24••], constructs with a mutated RXLR sequence accumulated GFP outside the malarial parasite but within the parasitophorous vacuole suggesting that the main function of the RXLR leader consists of transport across a host-derived membrane.

The structural similarity of the RXLR domain to the leader peptides of other protein transport systems suggests that comparable components may be required for the transport of RXLR effectors. Here, we suggest a model for effector delivery (illustrated in Figure 3) on the basis of the recurrent themes seen in these diverse protein targeting systems [29]. We propose that host translocation of RXLR effectors involves at least a RXLR leader binding protein, one or more additional chaperones, and a translocon, which could be of either pathogen or plant origin. Translocation into host cells initiates with the RXLR-binding protein recruiting mature effectors secreted via the general secretory pathway. In coordination with chaperones, the effector cargo is then transferred to a translocon embedded in the extrahaustorial membrane, and is then released across the membrane into the plant cytosol. The chaperones are important for maintaining the folding state of the transported effectors both before and after transit through the translocon.

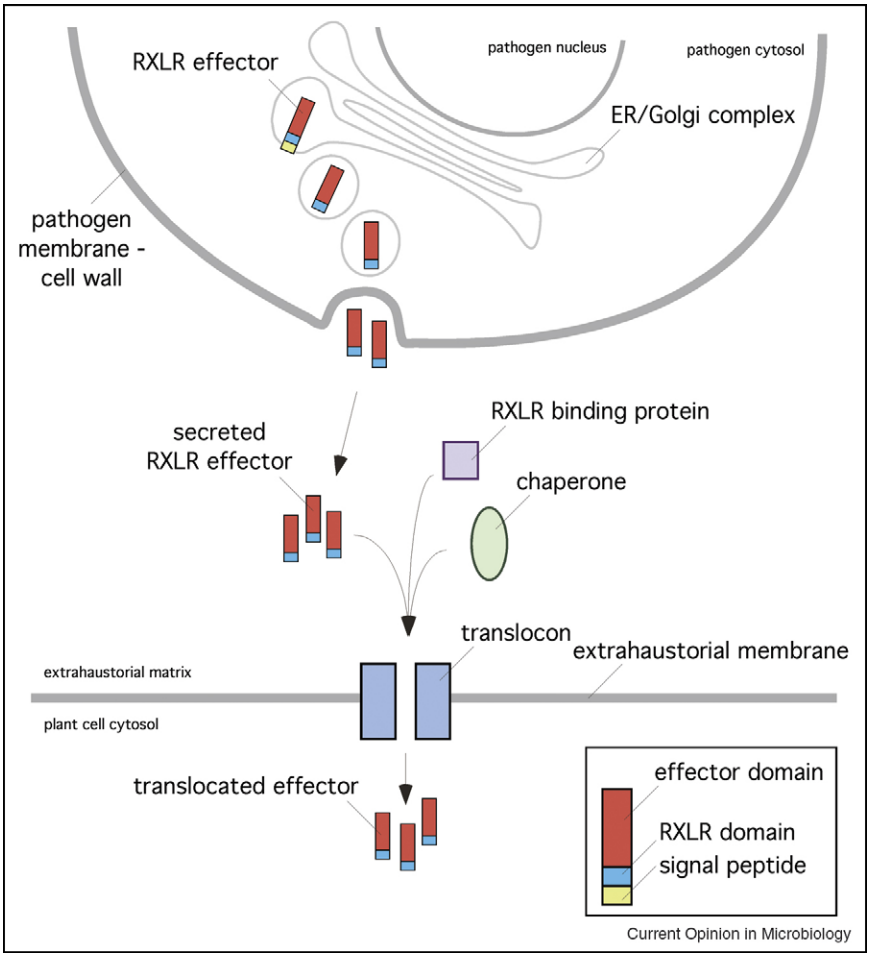
At this point, this model is highly speculative, but this outline provides a useful hypothesis generator to help guide future research. Indeed, the model suggests immediate research avenues that would shed light on the translocation process, for instance the identification of RXLR binding proteins and effector chaperones.

Virulence functions of RXLR effectors

Although the RXLR effectors were identified by virtue of their avirulence activity on plant cultivars with cognate *R* genes, presumably these effectors confer a selective advantage to the pathogen when infecting susceptible hosts. Consistent with this idea, overexpressing *Avr1b-1* increased pathogen virulence on a compatible host (D Dou *et al.* abstract 552, XXIV Fungal Genetics Conference, Pacific Grove, CA, March 2007). Unfortunately, the virulence function of RXLR effectors remains in great part unknown. Several RXLR effectors possess nuclear localization signals (NLS), suggesting that they manipulate host gene expression to secure an appropriate environment for infection [3,30].

Bos *et al.* [25••], in an effort to assign virulence-related functions to AVR3a, discovered that AVR3a^{KI} suppresses the hypersensitive cell death induced by the major *P. infestans* elicitin INF1 in *N. benthamiana*. The cell death suppression activity of AVR3a^{KI} exhibited some level of specificity. AVR3a^{KI} did not suppress the cell death induced by other *P. infestans* effectors, like PiNPP1 and CRN2, which elicit distinct and antagonistic cell death signaling pathways compared with INF1 [31]. The biological relevance of this activity of AVR3a^{KI} could be significant considering that suppression of innate immunity is a widespread function of biotrophic pathogen effectors, particularly the type III secretion system (TTSS) effectors of bacterial phytopathogens [32]. AVR3a^{KI} could interfere with the avirulence activity of INF1 or other unidentified effectors that trigger hyper-

Figure 3



A hypothetical model for RXLR effector secretion and delivery into host cells (see text for details).

sensitivity using similar pathways as INF1 [25••]. Future works are needed to clarify these issues and determine whether cell death suppression is a common function among RXLR effectors.

Outlook: too many effectors, too little time

Considering that genome sequencing and annotation is nearing completion for five oomycete species *H. parasitica*, *P. capsici*, *P. infestans*, *P. ramorum*, and *P. sojae*, we are moving rapidly toward genome-wide catalogs of RXLR effectors. Already it is evident that the RXLR effector secretome of plant pathogenic oomycetes is much more complex than expected, with perhaps several hundred proteins dedicated to manipulating host cells [3,15••]. Tyler *et al.* [15••] reported 350 RXLR effectors each in the genomes of *P. ramorum* and *P. sojae* using iterated similarity searches. Analyses in our own laboratories using combinations of motif and hidden Markov model searches uncovered at least 50 candidates in the downy mildew *H. parasitica* and more than 200 each in *P. capsici*, *P. infestans*, *P. ramorum*, and *P. sojae* (S Kamoun *et al.* abstract 188, XXIV Fungal Genetics Conference, Pacific Grove, CA, March 2007).

The task of tackling the study of so many effectors is daunting. One of the challenges is to establish ''effector-omics'' approaches, or global studies of effector function and activity. In addition to inferring function on the basis of homology and high throughput biochemical assays, other promising approaches include screens for suppressors of programmed cell death, phenotypic analysis of pathogen gene knockdowns, host transcriptional profiling in response to individual effectors, and three-dimensional structural determination of effectors (reviewed in [33,34]). Ultimately, comprehensive understanding of RXLR effector activities and the perturbations they cause in plants is crucial for understanding the molecular basis of oomycete pathogenesis and disease.

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